Table of Contents

§1. Experimental Section **1**

§2. Supplementary Schemes **10**

§3. Supplementary Figures **11**

§4. Supplementary Table **21**

§5. Full References used in the Main Manuscript **22**

§1. Experimental Section

Modified dendrimer synthesis: To synthesize modified dendrimers, poly(amido amine) and poly(propylenimine) dendrimers of increasing generations (Sigma Aldrich and SyMo-Chem) were reacted with alkyl epoxides (Tokyo Chemical Industry and Sigma) of various carbon chain length. The stoichiometric amount of epoxide added was equal to the total number of amine reactive sites within the dendrimer (2 sites for primary amines and 1 site for secondary amines). Reactants were combined in cleaned 20 mL amber glass vials. Vials were filled with 200 proof ethanol as the solvent and reacted at 90°C for 2 days in the dark under constant stirring. The crude product was mounted on a Celite™ 545 (VWR) pre-column and purified via flash chromatography using a CombiFlash Rf machine with a RediSep Gold Resolution silica column (Teledyne Isco) with gradient elution from 100% CH_2Cl_2 to 75:22:3 CH_2Cl_2:MeOH:NH_4OH_{aq} (by volume). Thin layer chromatography (TLC) was used to test the eluted fractions for the presence of modified dendrimers. Modified dendrimers with different levels of substitution appeared as a distinct band on the TLC plate when using a 87.5:11:1.5 CH_2Cl_2:MeOH:NH_4OH_{aq} (by volume) solvent system. All modified dendrimer-positive fractions were collected and dried under vacuum for final use. All products contained a mixture of conformational isomers.

Nanoparticle formulation: Tie2, FVII, scrambled Luciferase, CD45 and AFP siRNA were a gift from Alnylam Pharmaceuticals. Using 10 mM pH 3.0 citrate as a diluent, siRNA was loaded into a gas tight glass syringe. Modified dendrimers, $C_{14}PEG_{2000}$ (Avanti Polar Lipids) and the optional excipient cholesterol (Sigma) were dissolved in 200 proof ethanol and loaded into a second gas tight glass syringe. To form particles, each syringe was connected to the inlet ports of a single channel on a microfluidic mixing device containing static mixers.[1] The contents of the syringes were pumped into the channel at a 3:1

siRNA:modified dendrimer volumetric flow rate ratio and mixed within the channel to rapidly form well-defined nanoparticles. The resulting nanoparticles were dialyzed against sterile PBS using 20,000 molecular weight cutoff dialysis cassettes (Pierce Biotechnology) to remove ethanol and citrate before their sizes were characterized by dynamic light scattering using a Zetasizer Nano ZS machine (Malvern). For accurate dilutions and dosage, the concentration of siRNA was determined by a combination of Quant-iT™ Ribogreen® assay (Invitrogen) and NanoDrop measurement (Thermo Scientific).

In vitro gene knockdown screen: HeLa cells expressing firefly and Renilla luciferase were seeded at 14,000 cells per well into each well of an opaque white 96-well plate (Corning-Costar) and allowed to attach overnight at 37°C and 5% CO_2 in phenol red-free DMEM supplemented with 10% FBS (Invitrogen). Modified dendrimer nanoparticles were formulated with luciferase siRNA and cells were treated overnight at a 25 nM siRNA dose. Cells were then analyzed for luciferase expression using Dual-Glo assay kits (Promega) as previously described.[2] Luminescence was measured using an Infinite 200 PRO plate reader (Tecan).

Confocal imaging of nanoparticle uptake: HeLa cells were seeded at a density of 15,000 cells per well in a 96 well black Cellstar μClear Bottom plate (Greiner Bio One). Cells were treated with nanoparticles loaded with Cy5.5 labelled siRNA (Alnylam Pharmaceuticals) for 3 hours at a 50 nM dose. Cells were washed three times in PBS (Invitrogen), fixed for 20 minutes with 4% paraformaldehyde (EMS) and washed three times in PBS. Cell membranes were stained with Alexa Fluor 488 Wheat Germ Agglutinin (Life Technologies) and nuclei with Hoechst bisBenzimide H 33342 trihydrochloride (Sigma). After final PBS washes, all liquid was aspirated from the wells and replaced with Prolong gold anti-fade mounting medium (Life Technologies). Cells were imaged using a LSM 700 confocal microscope and a 60x oil emersion lens (Carl

Zeiss Microscopy). Images were acquired using z-stack imaging and presented as maximum intensity projections.

In vivo animal experiments: All procedures used in animal studies conducted at MIT were approved by the Institutional Animal Care and Use Committee (IACUC) and were also consistent with local, state and federal regulations as applicable.

FVII protein knockdown: Nanoparticles formulated with FVII siRNA were injected into the tail veins of 8 week old female C57BL/6 mice. After 48 hours, blood was collected from the animals and serum isolated with Falcon serum separation tubes (Becton Dickinson). To quantify the amount of protein knockdown induced by siRNA silencing, FVII protein levels were quantified in the serum using the Biophen FVII chromogenic assay (Aniara Corporation) according to the manufacturer's instructions. Relative FVII protein levels were calculated using a standard curve constructed from PBS-treated animals.

AFP protein knockdown: AFP silencing experiments were performed in mice with liver tumors 9 weeks after tumor induction via plasmid delivery, as described elsewhere.[3] Modified dendrimer nanoparticles formulated with AFP siRNA were injected into the tail veins of the mice and serum was collected 72 hours later. Western blotting was used to measure the serum levels of AFP protein. For each animal, a constant 0.5 µL volume of serum was heated for 20 minutes at 65°C in loading buffer and resolved on 7.5% TGX-PAGE gels (Bio-rad). Blots were transferred to nitrocellulose membrane (Bio-Rad), blocked in Odyssey blocking solution and probed overnight with primary AFP antibody (R&D systems). After incubation with

secondary antibodies (LI-COR Biosciences), membranes were imaged using a LI-COR Odyssey imaging system. Blots were quantified using ImageJ version 1.43u.

Tie2 gene silencing: Tie2 silencing experiments were performed in normal 8 week old female C57BL/6 mice (Charles River). Gene silencing was examined 48 or 72 hours after tail vein injection. Mice were euthanized by CO2 asphyxiation, organs harvested and immediately frozen in liquid nitrogen. Frozen tissues were pulverized to form a powder, and tissue lysates were prepared in Tissue and Cell Lysis Buffer (Epicentre) supplemented with 0.5 mg mL^{-1} Proteinase K (Epicentre). The mixture was mixed at 1400 RPM for 2 hours at 65°C and centrifuged at 16,000 RCF to remove any debris. The mRNA levels in the supernatant (lysate) was quantified using the QuantiGene 2.0 luminescent-based branched DNA assay kit and QuantiGene 2.0 probes against tie2 and gapdh (Affymetrix) according to the manufacturer's protocol. Luminescent signal was measured with a Tecan Infinite 200 PRO plate reader. To avoid signal saturation and to ensure all luminescent signals remained within their linear regions, a standard curve for each tissue and target gene was constructed using samples from PBS-treated mice to determine the optimal dilutions for assay samples. The relative silencing in treated groups was determined by calculating the ratio of target gene luminescence to gapdh housekeeper gene luminescence. All values were normalized to the target:housekeeper gene ratio from PBS-treated mice.

CD45 knockdown: 8-10 week old, female C57BL/6 mice (Charles River) were intravenously injected via the tail vein with CD45 siRNA-carrying nanoparticles at 1 mg kg^{-1}. After 3 days, spleens were harvested and mechanical disrupted using a nylon mesh (cell strainer, BD Falcon) in 10 ml of DMEM, 10% FBS. Spleen cell suspension was treated with ACK lysing buffer (Life Technologies), washed twice and resuspended in PBS, 4% FBS, 0.02% sodium azide. Peritoneal cavity leukocytes were recovered by washing

the peritoneal cavity with 4 mL of PBS, and cell suspensions were washed with PBS, 4% FBS, 0.02% sodium azide. Fluorescence staining was performed with a combination of the following mouse-specific antibodies: CD45-FITC, GR1-PE, CD11b-PE/Cy7, CD11c-APC, CD19-PE, TCRβ-APC and CD32/16 (Biolegend). Cells were analyzed by flow cytometry (BD LSR II HTS).

Cytokine screen: Blood samples were collected at 2 days following tail vein injection of modified dendrimer nanoparticles in C57BL/6 mice, and plasma was isolated for cytokine array screening using the Bio-Plex Protein Array System (Bio-Rad) with a Luminex 200 instrument. The Bio-Plex ProTM Mouse Cytokine 23-plex panel was combined with the Bio-Plex ProTM Mouse Cytokine 9-plex panel for an array totaling 32 cytokines. The assay was performed according to manufacturer's instructions. Plasma was diluted 1:4 in the sample diluent buffer provided, and all cytokines were quantified using a known standard sample containing a dilution series of all 32 cytokines. Unique beads containing capture antibodies for the following cytokines were used: interleukin (IL) family members (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IL-18), Eotaxin (CCL11), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), interferon γ (IFN-γ), keratinocyte-derived chemokine (KC, CXCL1), monocyte chemotactic protein 1 (MCP-1, CCL2), macrophage inflammatory protein 1α (MIP-1α, CCL3), MIP-1β (CCL4), MIP-2 (CXCL2), Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES, CCL5), tumor necrosis factor α (TNF-α), basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), monokine induced by gamma interferon (MIG, CXCL9), platelet-derived growth factor-BB (PDGF-BB), and vascular endothelial growth factor (VEGF). Protein concentration in each sample was determined using specific biotinylated secondary antibodies, followed by PE-streptavidin to quantify protein levels. At minimum, 50

beads were counted for each cytokine in each sample. Raw concentration in pg mL^{-1} were obtained for each cytokine in each sample and expressed relative to a control group receiving a PBS injection.

^{1}H NMR: 5 mg of modified dendrimer was dissolved in 2 mL of deuterated chloroform ($CDCl_3$, Cambridge Isotope Laboratories, Inc.) with a solvent peak at 7.26 ppm. Spectra were recorded on a 600 MHz Bruker AVANCE-600 NMR machine.

Determination of apparent nanoparticle pK_a via TNS assay: The pK_a of fully formulated nanoparticles, including all excipients and negatively-charged siRNA, was determined via 6-(p-toluidino)-2-naphthalenesulfonic acid (TNS, Invitrogen) assay. The advantage of this method was that nanoparticle structure and formulation were taken into account. Thus, the measured pK_a is that of the entire nanoparticle in an aqueous environment similar to delivery conditions, and not the individual modified dendrimer molecule.[4] Potentiometric titration, the alternative method for determining ionization constants, was not suitable for this study because it is incompatible with fully formulated nanoparticles, as it requires the tested materials to be fully solubilized and dissolved in solution, which is not possible for colloidal suspensions.[4a] A stock buffer solution containing 10 mM HEPES (Sigma), 10 mM MES (Sigma), 10 mM sodium acetate (Sigma) and 140 mM sodium chloride (Sigma) was prepared. Using this stock solution and 1.0 N HCl or NaOH, pH adjusted solution from pH 2.0 to pH 12 were prepared in pH increments of 0.5. Using quadruplets for each type of nanoparticle, 140 µL of pH-adjusted solution was added to the wells of a 96 well plate, followed by 5 µL of 60 µg mL^{-1} of TNS. As the last step, 5 µL of 0.3 mg/mL (modified dendrimer concentration) nanoparticles was added to the appropriate wells. The plate was shaken at 350 RPM at room temperature for 5 minutes. After being placed in a Tecan Infinite M200 microplate reader and shaken for an additional 2 minutes at room temperature (1mm amplitude), the fluorescence was measured using excitation

and emission wavelengths of 325 and 435 nm, respectively. The fluorescent signal was plotted against the pH to generate a curve. The pH value at which half of the maximum fluorescence was reached was reported as the apparent nanoparticle pK_a.

Nanoparticle serum stability Fluorescence Resonance Energy Transfer (FRET) assay: siRNA duplexes labelled at the 5'end of the sense strand with either Alexa Fluor594 or Alexa Fluor647 dyes were purchased from Integrated DNA Technologies. The siRNAs were HPLC purified and de-salted. Their sequences were:

siRNA #1 sense strand: 5'-Alex594-GAUUAUGUCCGGUUAUGUAUU-3'

siRNA #2 sense strand: 5'-Alex647-GAUUAUGUCCGGUUAUGUAUU-3'

Antisense strand common to both siRNA sense strands: 5'-UACAUAACCGGACAUAAUCUU-3'

Nanoparticles with equimolar amounts of both siRNAs were formulated and diluted to a final concentration of 0.002 mg mL^{-1} siRNA. In quadruplets, 100 µL of the diluted nanoparticles were added each well of an opaque black 96 well plate. 100 µL of 50% AB human serum (Invitrogen), which had been diluted in PBS, was added to each well. Negative control wells contained free siRNA. Positive control wells contained PEI nanoparticles. PEI nanoparticles were formed by the repeat pipetting of 800 MW PEI (Sigma) with siRNA in a 5:1 PEI:siRNA mass ratio in a 10 wt% sucrose solution. The plate was sealed with a clear adhesive plate seal and placed into a Tecan Infinite M200 microplate reader set to 37°C. To measure FRET, samples were excited at 540 nm and the fluorescent intensity was read at 690 and 620 nm every 5 minutes for 2 hours. FRET was calculated as the 690nm/620nm fluorescent intensity signal ratio. FRET values were normalized to the values of the completely ruptured nanoparticle, which were determined after adding octyl β-D-glucopyranoside (Sigma) to a final in-well concentration of 2 wt% and mixing for 1 hour at 37°C.

Zeta Potential: 25 µL of formulated nanoparticles at with a modified dendrimer concentration of 1 mg/mL was added to 1 mL of PBS and zeta potential measurement were determined using a Malvern Zetasizer Nano ZS machine.

Biodistribution: Modified dendrimers were formulated with Cy5.5 labelled siRNA and injected into the tail veins of C57BL/6 mice. After 1 hour, mice were euthanized, organs harvested and imaged using an IVIS Spectrum-bioluminescent and fluorescent imaging system (Xenogen Corporation).

Statistical analysis: The student t-test was used to gauge statistically significant differences in mean values. P values less than 0.05 were considered statistically significant. Statistical analysis was performed using version 16 of the SPSS statistics software package.

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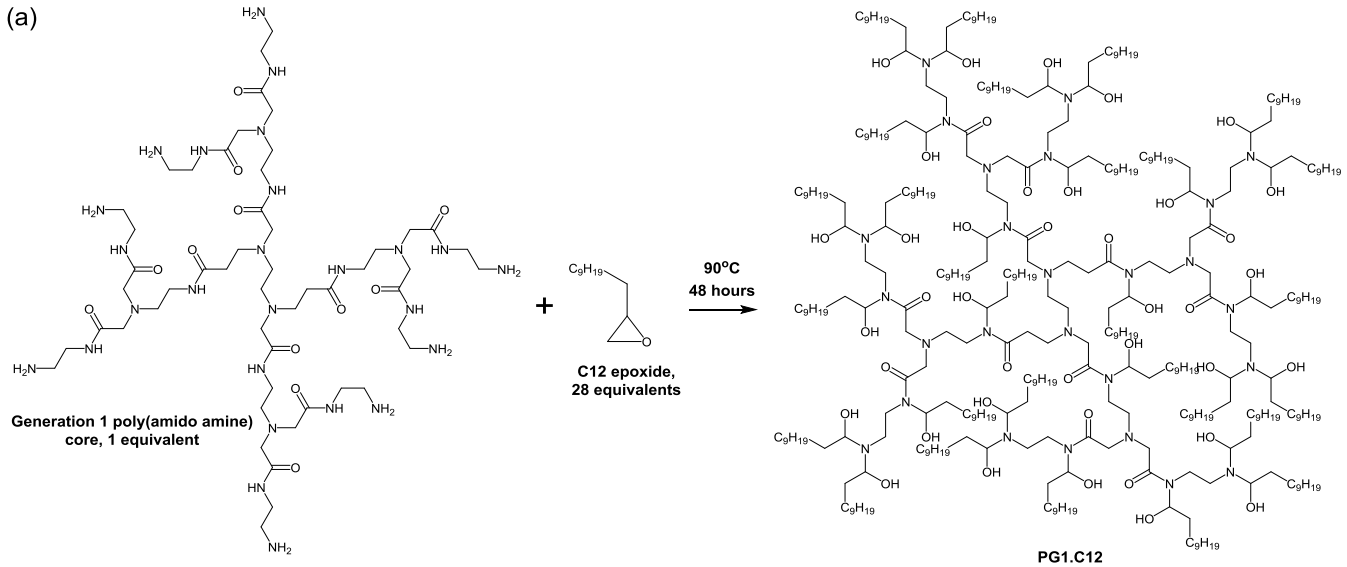
§2. Supplementary Schemes



Scheme S1. (a) Synthesis of PG1.C12 from generation 1 poly(amido amine) dendrimer and C12 epoxide. (b) Synthesis of PG1.C15 from generation 1 poly(amido amine) dendrimer and C15 epoxide.

§3. Supplementary Figures

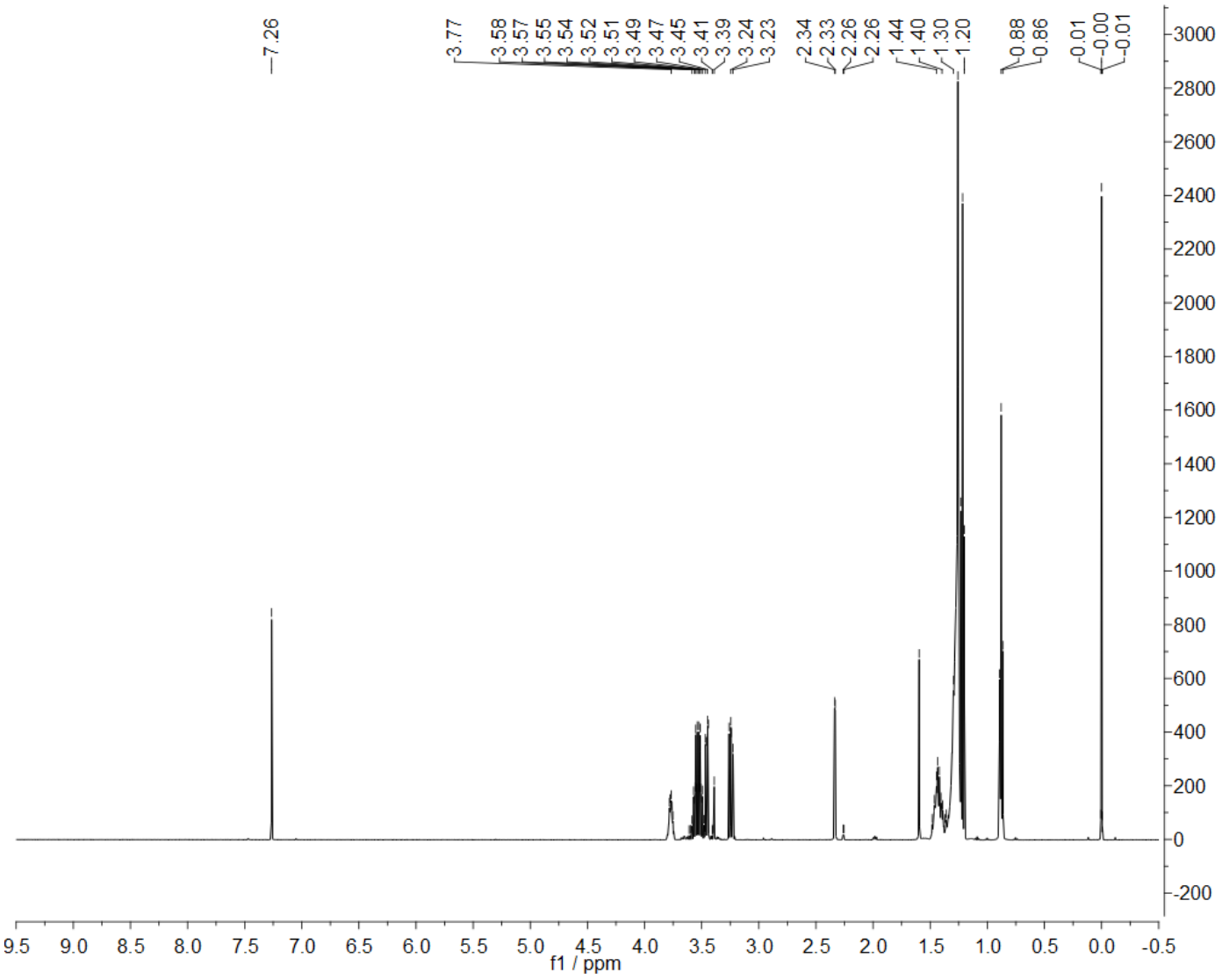


Figure S1. ^{1}H NMR for PG1.C12. The $CDCl_3$ solvent peak appears at 7.26 ppm.

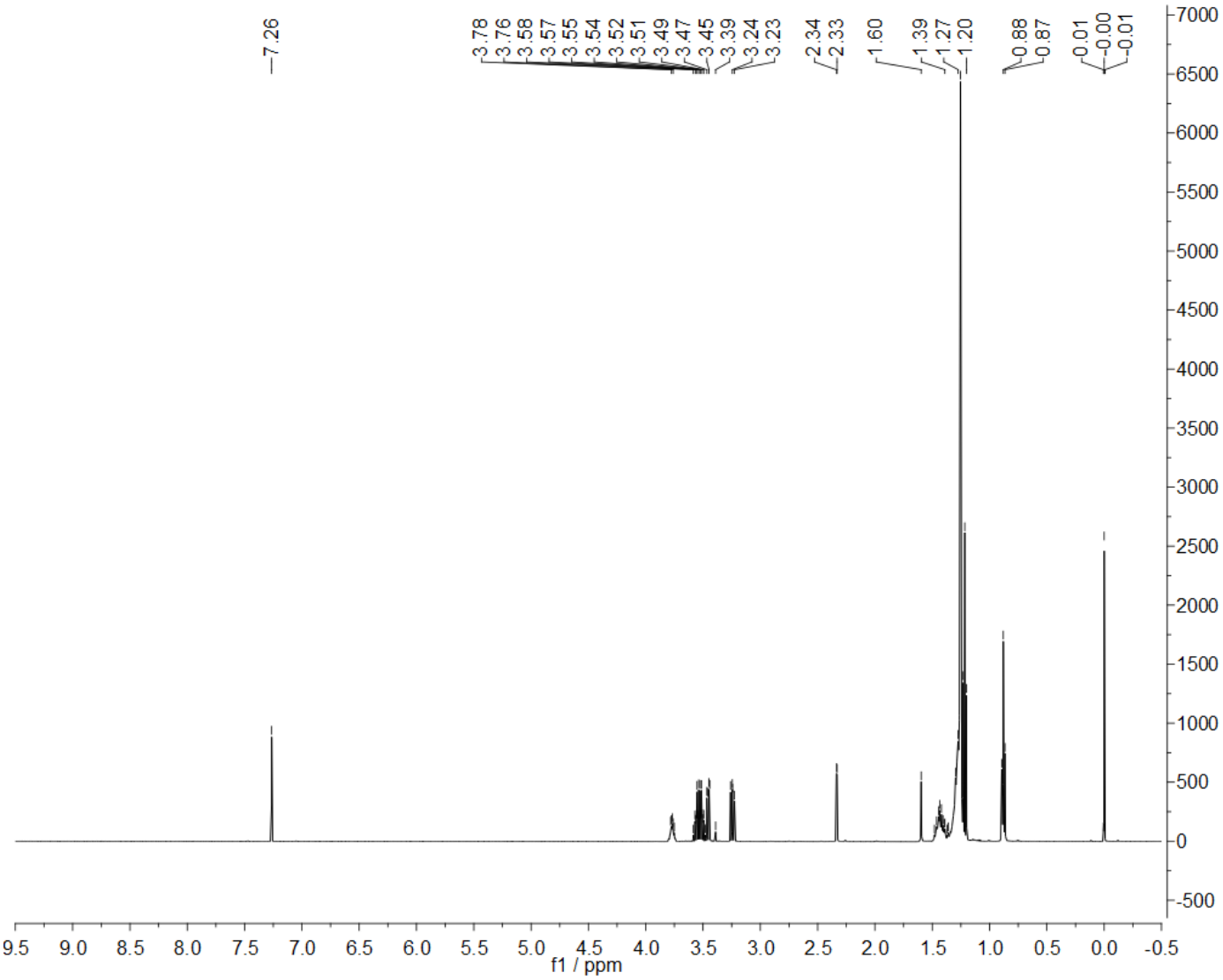


Figure S2. ^{1}H NMR for PG1.C15. The $CDCl_3$ solvent peak appears at 7.26 ppm.

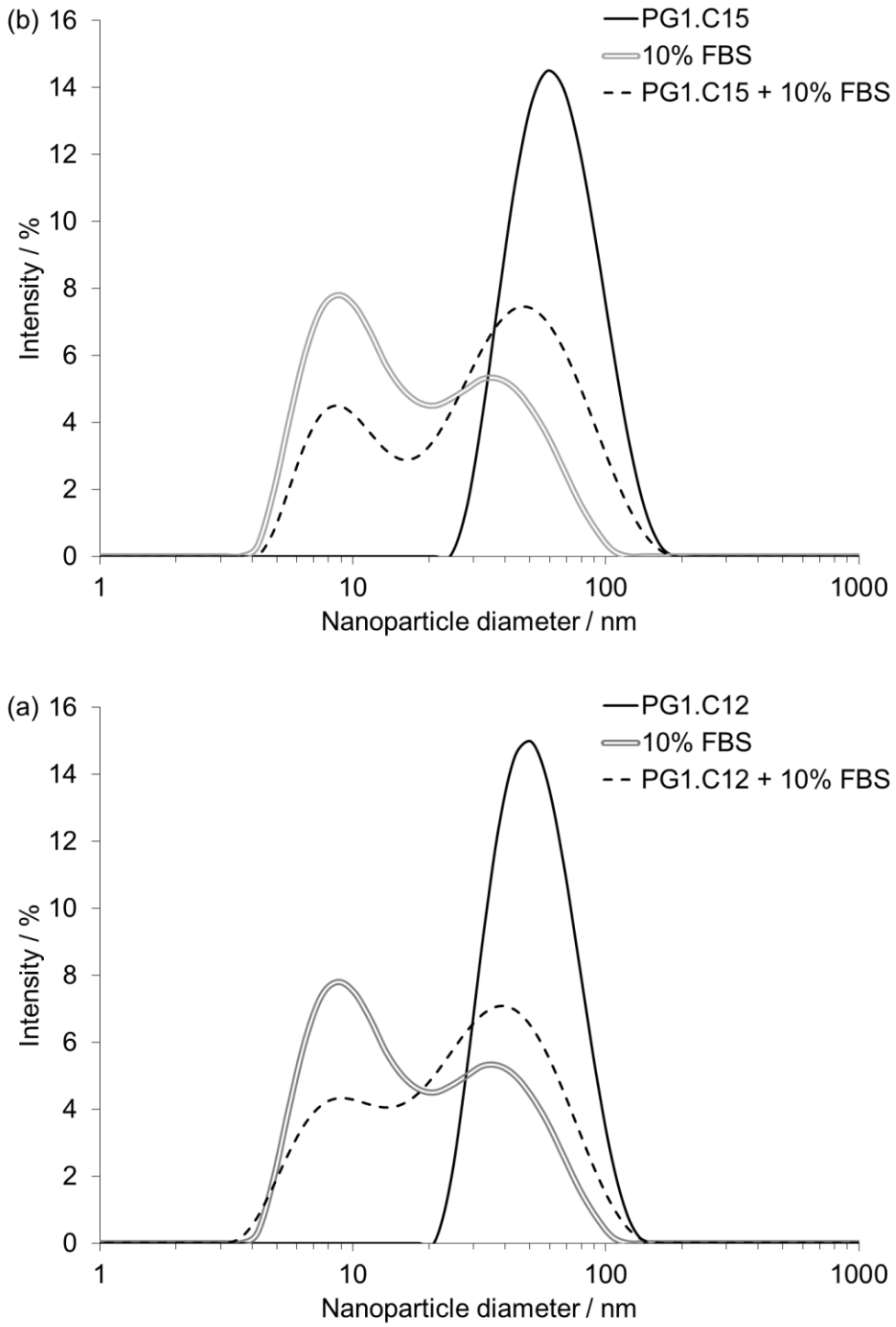


Figure S3. The effect of serum on nanoparticle size. Nanoparticle diameter (by intensity) in PBS was measured before (solid line) and after (dashed line) the addition of fetal bovine serum (FBS). FBS alone appears as a double grey line. All FBS-containing samples had a final serum concentration of 10% v/v in PBS. (a) PG1.C12. (b) PG1.C15.

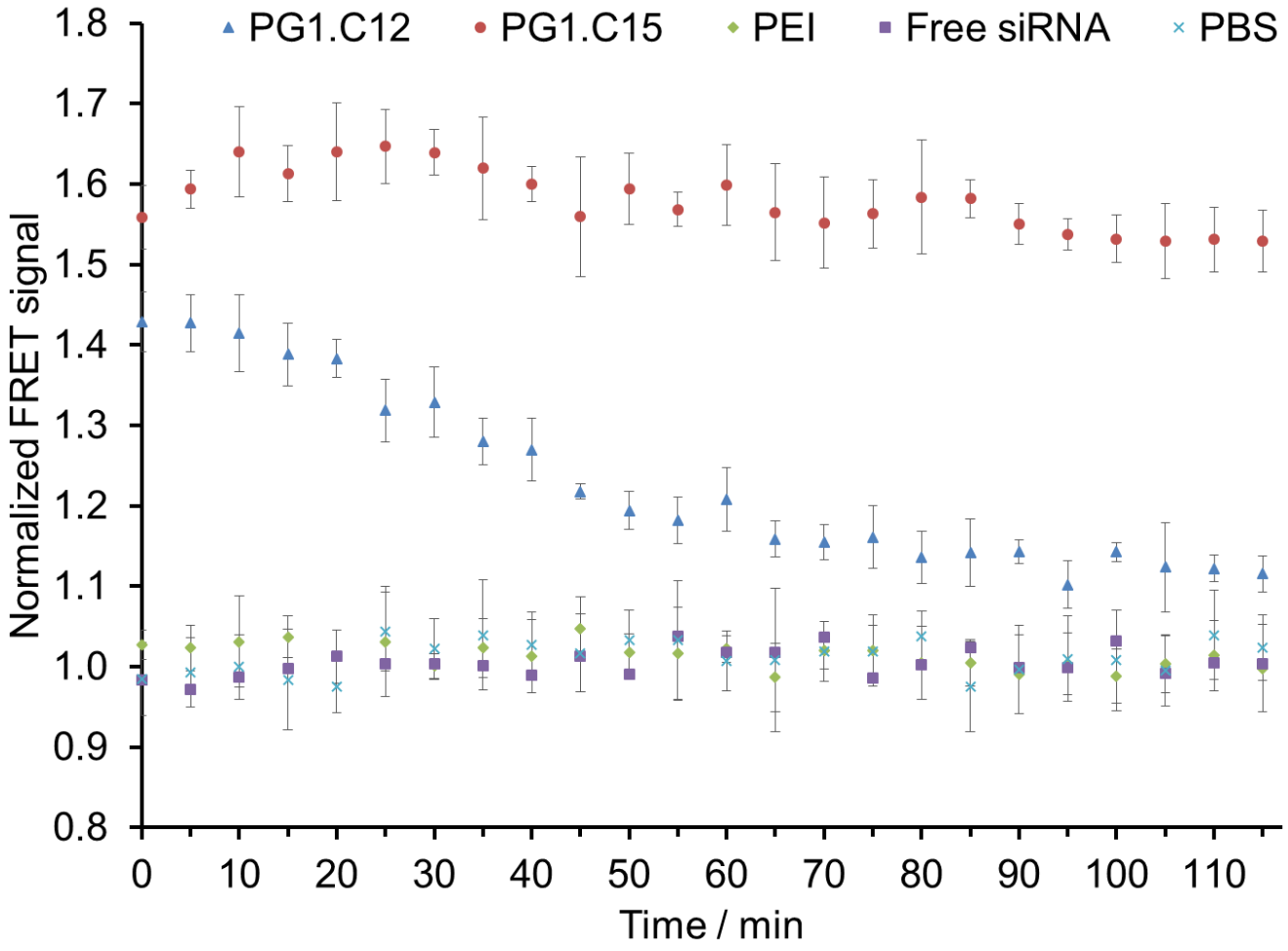


Figure S4. Stability of formulated PG1.C12 and PG1.C15 nanoparticles over 2 hours in 50% AB human serum at 37°C using a Fluorescence Resonance Energy Transfer (FRET) assay. The mass ratio of modified dendrimer to siRNAs was 5 to 1 and the nanoparticles contained $C_{14}PEG_{2000}$ as an excipient. Negative controls were free siRNA. PBS was used to determine background levels. PEI complexes were used for additional comparison. FRET signal was normalized to the value of the completely ruptured nanoparticles. Intact nanoparticles that still contained their siRNA payloads had higher FRET signals, which contrasted with the low FRET signals seen in the free siRNA negative controls. N = 4 and error bars ± S.D.

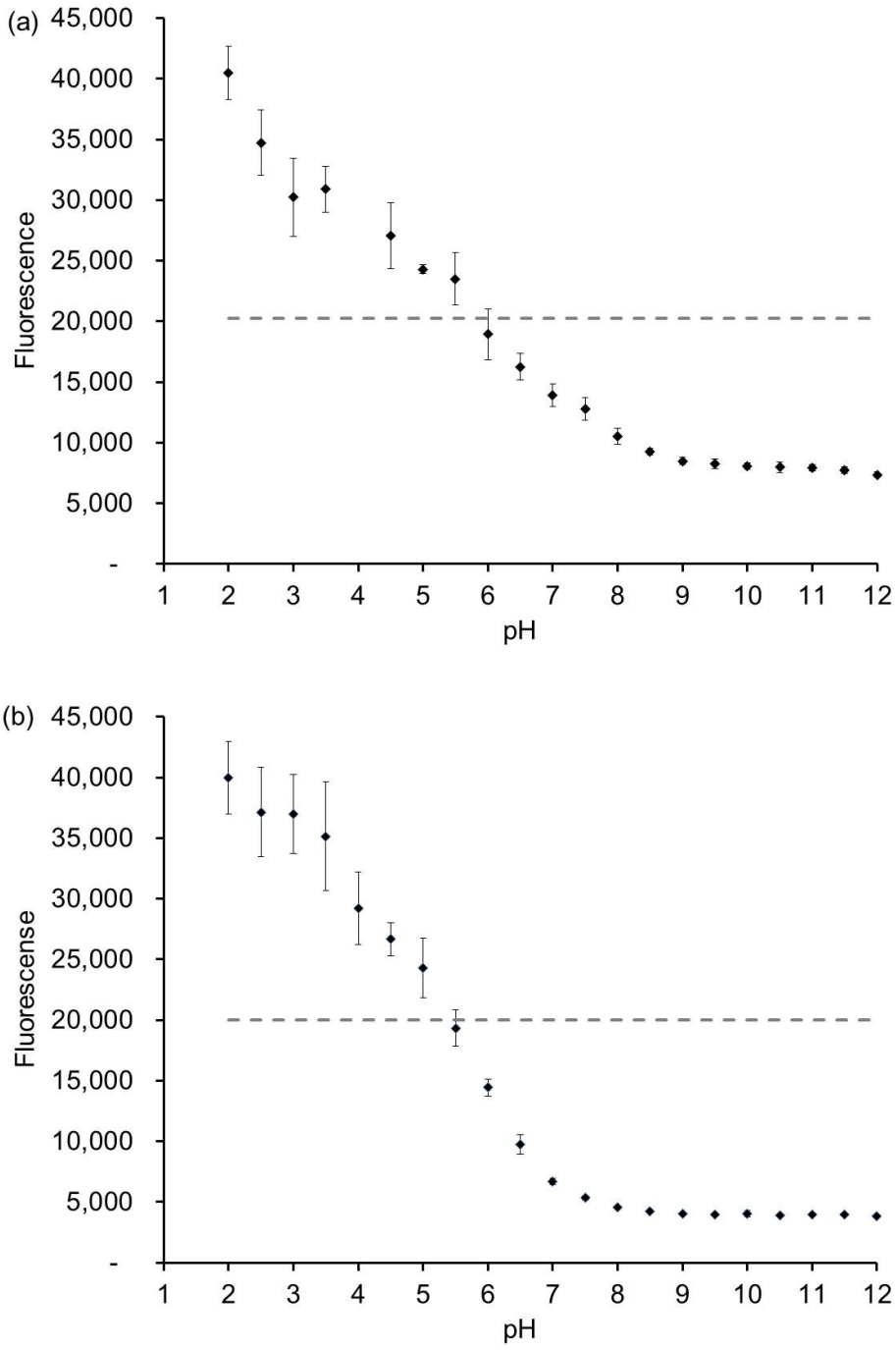


Figure S5. Apparent pK_a values for fully formulated (a) PG1.C12 and (b) PG1.C15 nanoparticles, as determined by 6-(p-toluidino)-2-naphthalenesulfonic acid (TNS) assay. The modified dendrimer:siRNA mass ratio was 5:1 and the nanoparticles contained $C_{14}PEG_{2000}$ as an excipient. The dashed grey line represents half of the maximum fluorescent intensity, which intersects the curve at the apparent nanoparticle pK_a. N=4 and error bars are ±S.D.

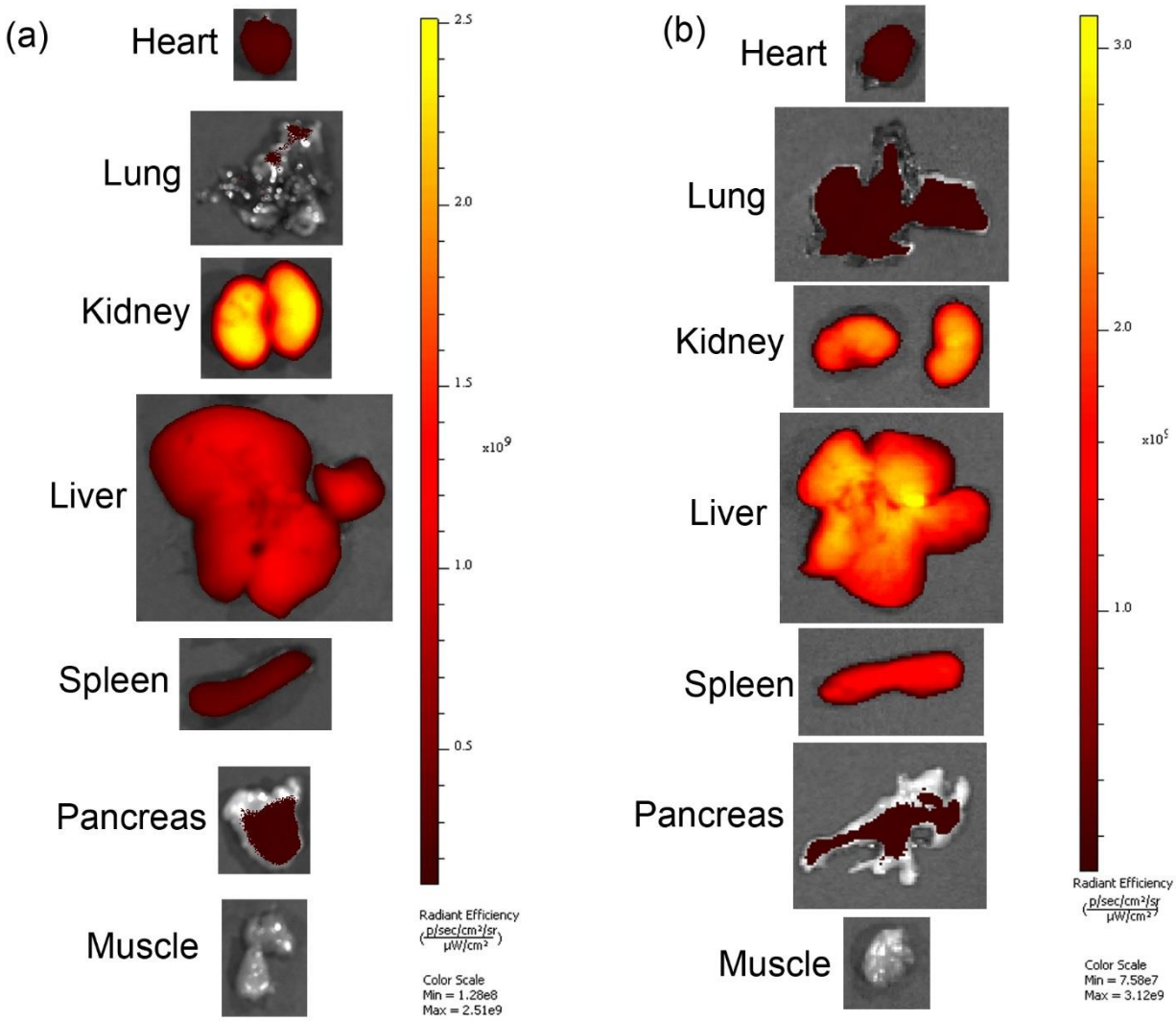


Figure S6. Biodistribution of (a) PG1.C12 and (b) PG1.C15 nanoparticles formulated with Cy5.5 labelled siRNA 1 hour after injection. The modified dendrimer:siRNA mass ratio was 5:1 and the nanoparticles contained $C_{14}PEG_{2000}$ as an excipient. Three animals were used to examine the nanoparticle biodistribution and representative images from one mouse are shown for each modified dendrimer. The high signal in the kidneys was due to nanoparticle clearance from the blood and into urine.

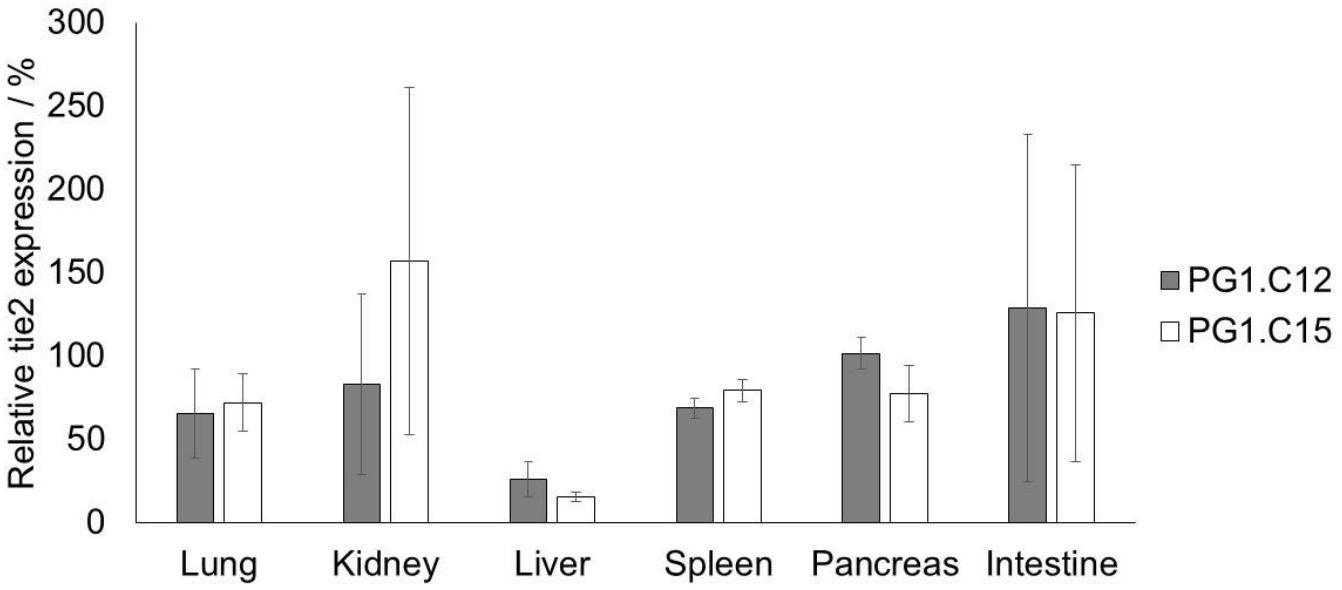


Figure S7. The PG1.C12 and PG1.C15 lead materials were examined for endothelial cell tie2 knockdown in the lung, kidney, liver, spleen, intestine and pancreas of mice. These materials were formulated with tie2 siRNA and $C_{14}PEG_{2000}$, and injected in the tail veins of mice at a dose of 2.5 mg kg^{-1}. After 2 days, knockdown was most efficacious in the liver endothelium. N = 3 and error bars are ± 1 S.D.

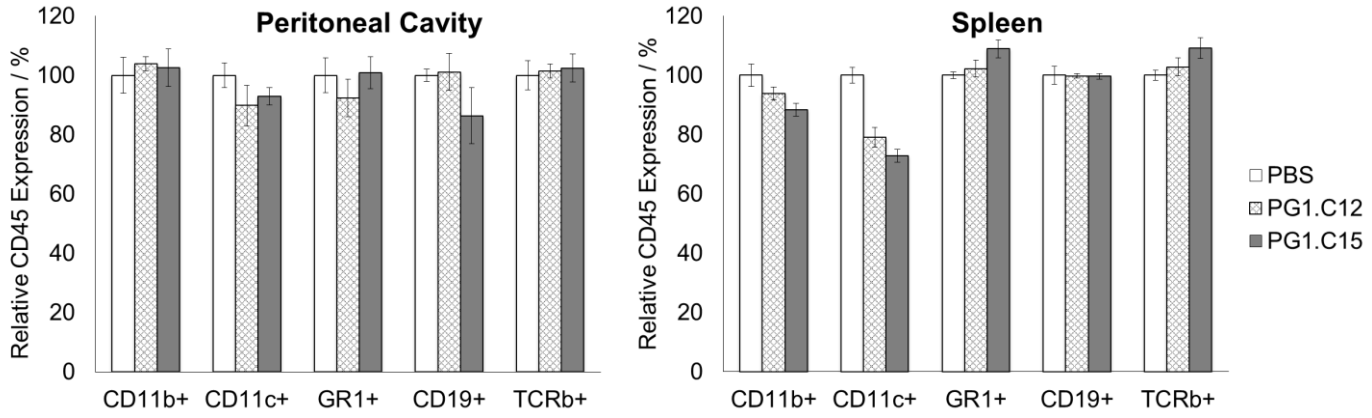


Figure S8. To determine if modified dendrimers were targeting immune cell populations, nanoparticles were formulated with siRNA against the pan-leukocyte marker CD45 and used to treat mice at a 1 mg kg^{-1} dose. Immune cells from the peritoneal cavity and spleen were isolated and CD45 knockdown was assessed by flow cytometery. Immune cells were identified by CD11b (macrophage/monocyte), CD11c (dendritic cells), GR1 (granulocytes), CD19 (B cells) and TCRb (T cells) markers. With the exception of limited knockdown in CD11c-positive cells from the spleen, no major knockdown was observed. N = 3 and error bars are ± 1 S.D.

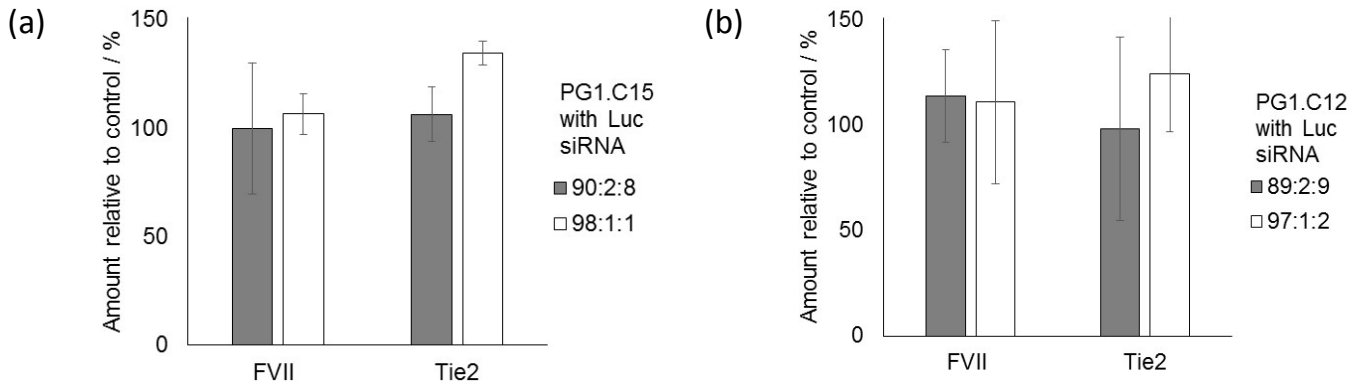


Figure S9. As negative controls, optimized nanoparticles containing cholesterol and $C_{14}PEG_{2000}$ as excipients were formulated with non-functional scrambled Luciferase (Luc) siRNA at a 2.5 mg kg^{-1} dose. The modified dendrimer:cholesterol:$C_{14}PEG_{2000}$ mass ratios are shown in the figure legends. Two days after the nanoparticles were injected into the tail veins of mice, no FVII or tie2 knockdown was observed for PG1.C15 **(a)** and PG1.C12 **(b)** treatments. N = 3 and error bars are ± S.D.

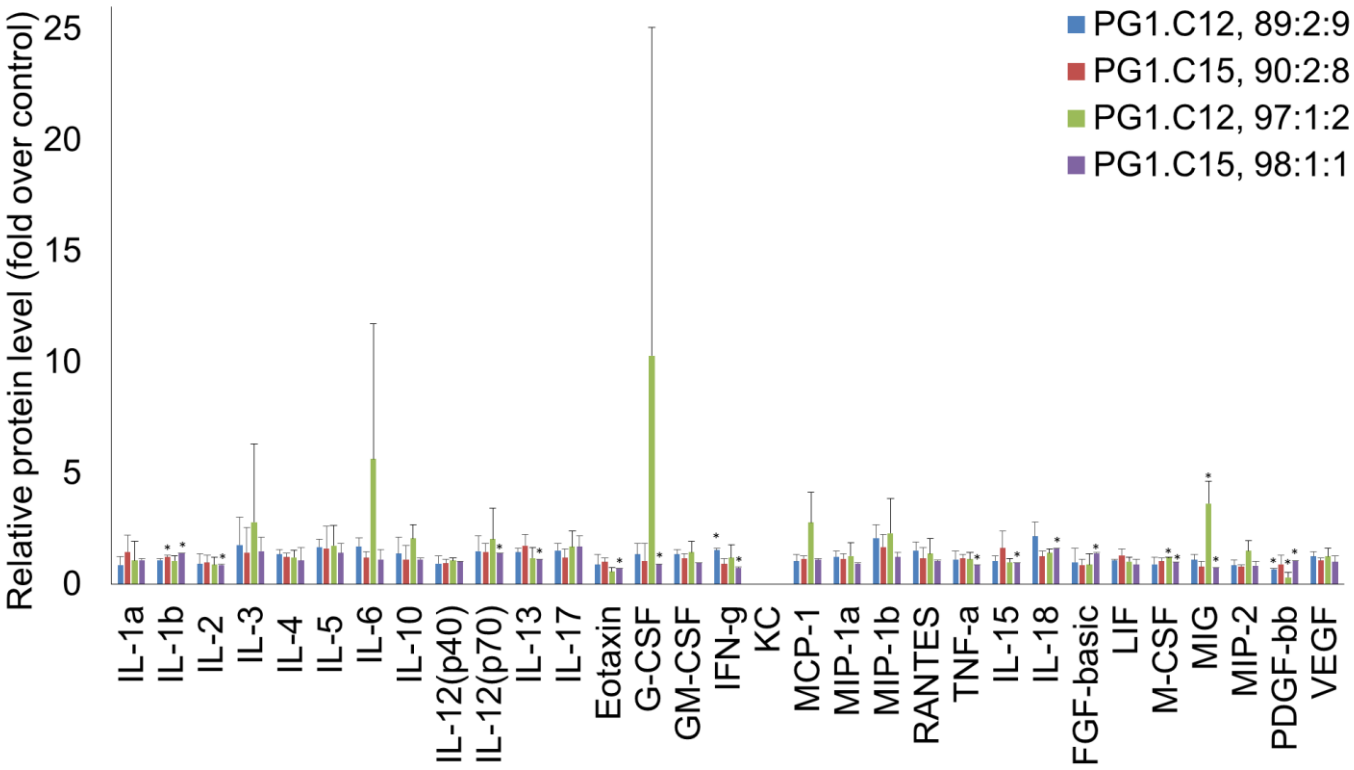


Figure S10. Inflammatory cytokine screen in mouse plasma 48 hours after treatment. To determine if the modified dendrimer-based nanoparticles caused any long-term chronic inflammatory effects, nanoparticles were formulated with non-functional scrambled Luciferase siRNA and injected at a dose of 2.5 mg kg^{-1}. No dramatic increase in blood plasma cytokine levels were observed; however, formulations with higher cholesterol content resulted in fewer fluctuations in cytokine levels relative to baseline. Error bars are + 1 S.D. N was between 3 and 6 biological replicates. * indicates a fold change significantly different than 1 ($p < 0.05$).

§4. Supplementary Table

Table S1. Zeta potential and pK_a values for formulated PG1.C12 and PG1.C15 nanoparticles. The modified dendrimer:siRNA mass ratio was 5:1 and the nanoparticles contained $C_{14}PEG_{2000}$ as an excipient. For zeta potential measurements, the average value from quadruplet samples are presented ± S.D.

Modified Dendrimer	Zeta Potential / mV	pK_a
PG1.C12	-15.0 ± 3.6	5.9
PG1.C15	-10.7 ± 1.8	5.5

§5. Full References used in the Main Manuscript

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